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Multipotential Cells in the Bone Marrow Stroma: Regulation in the Context of Organ Physiology

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ABSTRACT: Multipotential (osteogenic, adipogenic, chondrogenic, and myelosupportive) cells associated with the bone marrow stroma are revealed by *in vitro* or *in vivo* differentiation assays. If considered in the context of development, growth, and adaptive changes of bone as an organ, the hierarchical organization, histophysiology, and biological significance of the so-called "stromal system" appear distinct from those predicted from the commonly used analogy with the hematopoietic system, with which the stromal system and its putative "stem" cell are usually compared. The plasticity of differentiated phenotypes and the emergence of individual lineages in a defined temporal succession throughout development and postnatal life reflect the role of the multipotential cells in the stromal system in tissue adaptation and growth, rather than in cell consumption and replacement. This makes the stromal system and its progenitors an interesting paradigm of the biology of an individual cell's flexibility in complex organisms.

KEY WORDS: marrow stromal cells, stem cells, osteogenesis, commitment, adipogenesis, development.

I. INTRODUCTION

Many reasons underlie the currently exploding interest in the biology of marrow stromal cells. First, the very idea of a lineage relationship between different skeletal tissues and a putative common progenitor is highly appealing. Second, this view provides a new angle for reading pathological changes in the skeleton (Bianco and Robey, 1999). Third, the multipotential nature of marrow stromal cells provides an option for developing novel means of therapeutic intervention in skeletal diseases, including somatic cell therapy and gene therapy (Prockop, 1997). Finally, the glimpse provided by recent studies of the potential of new facets of marrow stromal cell biology offers encouragement for their ultimate use in systemic transplantation (Horwitz et al., 1999; Nilsson et al., 1999); however, this postulated application is somewhat controversial (and is not discussed here).

The current flurry of activity in the field stems from the pioneering work of several far-sighted individuals, and the conceptual framework that they originally defined (Friedenstein et al., 1968; Friedenstein et al., 1966; Owen, 1988; Owen and Friedenstein, 1988). As is often the case in human activities, ideas that are new and propulsive at the time they are set forth, later become crystallized in widely held beliefs that are often oversimplified and sometimes incorrect. As many think of the stromal system and its "stem cells," their minds rush to the hematopoietic system paradigm, from which the concept was once borrowed for the sake of effective conceptualization. Yet, as we learn of the great diversity of stem cells and systems that depend upon them (Morrison et al., 1997), we should also recognize how divergent the biology of different "stem" cells must be. By overlooking the general context of skeletal and marrow biology, potentially unique aspects of the

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putative "stromal stem cells" may be overlooked, and their full potential may not be utilized, even in biotechnology.

II. THE IDEA OF A LINEAGE AND THE LINEAGE OF AN IDEA

Curiously, the first indication that bone could be formed from marrow dates back to a time when it was still unknown that marrow cells replenish the blood cell population throughout life. Goujon observed that transplants of marrow fragments in the abdominal cavity resulted in formation of ectopic bone in 1866, before Neumann established in 1868 that red blood cells were formed in the marrow (reviewed in Tavassoli and Yoffey, 1983). The idea was premature for the times, and it was not revived until the late 1960s.

Friedenstein and coworkers showed that bone marrow stromal cells formed bone and marrow tissues when transplanted in an appropriate environment (Friedenstein et al., 1968; Friedenstein et al., 1966). Subsequently, he assigned the ability to completely regenerate a bone/bone marrow organ to a population of clonogenic adherent cells of nonhematopoietic origin, the colony forming unit-fibroblast (CFU-F) (Friedenstein et al., 1976). The progeny of the CFU-F (bone marrow stromal cells [BMSCs]) share some, but not all, characteristics of fibroblastic cells of other tissues. Although several features are shared with endothelial cells, they lack the basic characteristics of this cell type (Factor VIII production and the Weibel-Palade body) and are totally devoid of features characteristic of macrophages (Castro-Malaspina et al., 1980; Fei et al., 1990; Song and Quesenberry, 1984; Zhang et al., 1995). Further studies by Friedenstein, and Owen and coworkers were based upon *in vivo* transplantation (the gold standard of phenotypic characterization) using open systems (subcutaneous or under the kidney capsule) or closed systems (diffusion chamber). These studies demonstrated that BMSCs maintain the ability to form bone, cartilage, fibrous tissue, hematopoiesis-supporting reticular stroma, and associated adipocytes (Ashton et al., 1980; Bennett et al., 1991; Chailakhyan et al., 1978; Owen, 1988; Patt et al., 1982). Using genetic markers, the bone and associated stroma were found to be of donor origin, whereas the hematopoiesis supported by these tissues is of recipient origin (Friedenstein and Kuralesova, 1971; Friedenstein, 1980; Friedenstein et al., 1978). More recent studies have focused on biochemical characterization of these cells by *in vitro* and *in vivo* analyses. The sum total of these studies has reconfirmed the original observations that the stromal cell system contains a population of multipotential cells that have the ability to completely regenerate a bone/bone marrow organ upon *in vivo* transplantation (Dennis and Caplan, 1996; Goshima et al., 1991; Krebsbach et al., 1997; Kuznetsov et al., 1997b; Ohgushi and Okumura, 1990).

The ability of marrow stromal cells to differentiate into diverse tissues comprised in bone as an organ (bone, cartilage, adipocytes, fibroblasts, and myelosupportive stroma) led to the hypothesis that stromal cells comprised a multipotent progenitor conceptually akin to the hematopoietic stem cell. The analogy was natural, in view of the common medullary source of the two systems as well as the general paradigm of a diversified system of lineages emanating from a common ancestor that the hematopoietic system provides.

III. UNIQUE CHARACTERISTICS OF THE STROMAL SYSTEM

Important differences distinguish the inherent dynamics of the hematopoietic and stromal systems.

1. Evidence that stromal "stem" cells can replenish the tissues that depend on them throughout the lifetime of an organism is missing. Aging per se involves bone loss and, in a variety of animal species, CFU-F (of which the putative stem cell must be a member) decrease in number with age (Jilka et al., 1996; Quarto et al., 1995; and Kuznetsov and Gehron Robey, unpublished results). In contrast, blood cell turnover does not attenuate significantly with aging. In bone marrow transplantation, after lethal irradiation, stem cells reconstitute hematopoiesis for an individual's lifetime.

2. Tissues (lineages) formed within the stromal system are not synchronous, but appear at distinct stages of pre- or postnatal development and growth (reviewed in Bianco and Riminucci, 1998). For example, adipogenesis in the marrow is an entirely postnatal event. Chondrogenesis is the earliest event of embryonic bone development, but simply never happens within the postnatal marrow, either from marrow stromal cells or any other cell, other than in fracture repair. Dense fibrous tissue is found in the periosteum, but not within the marrow, where it is only formed in disease. In contrast, red blood cells, platelets, and granulocytes are formed continuously and at the same time.
3. Tissues within the stromal system are solid-phase and grow in size and cell number. Blood is fluid, and does not grow in size and cell number with organism growth.
4. Once growth has ceased, skeletal tissues turn over at a much slower rate than blood cells. All granulocytes are completely replaced over 450 times in a human adult's lifetime. Direct measurements of bone formation rates predict that a bone mass equivalent to the whole skeleton is only turned over twice in the same time period. This is an average estimate, as some sites (e.g., the linea aspera of the femur) turn over continuously while other skeletal sites may never turn over in a lifetime. Furthermore, in small mammals, a true Haversian remodeling does not occur and cortical bone of a mouse femur, for example, is only subject to growth-related modeling.
5. Tissues (lineages) formed within the stromal system are not rigidly separated downstream of the putative stem cell. Examples of this are provided by the ability of "terminally" differentiated chondrocytes to switch to an osteoblast-like phenotype (Galotto et al., 1994; Gentili et al., 1993), by the ability of marrow adipocytes to revert to an osteogenic capacity (Bennett et al., 1991; Beresford et al., 1992), and by the evidence that these events occur not only in culture, but also *in vivo* (Bianco et al., 1988; Bianco et al., 1998; Riminucci et al., 1998). This is in stark contrast to the hematopoietic system, where it is not possible to convert a red blood cell into a platelet.

Marrow stromal "stem" cells function in organ growth and repair but do not continuously replenish a compartment of differentiated cells that undergo rapid turnover. Most likely they also provide the reservoir for newly differentiated osteoblasts that accomplish postnatal turnover in humans. However, whether this requires the intervention of a true stem cell, rather than the simple recruitment of committed progenitors, is not known. Furthermore, bone turnover is not restricted to trabecular bone, which interfaces with the bone marrow. It also involves bone surfaces not in contact with marrow, such as the outer surfaces or intracortical Haversian systems. Here, osteoblasts must come from local progenitors that are not located in the marrow. Hence, the dominant commonplace statement evolved from the discovery of multipotential stromal cells, that "osteoblasts derive from the marrow," is an unwarranted oversimplification.

IV. DEFINITION AND IN SITU IDENTITY OF BONE MARROW STROMAL CELLS

Marrow stromal cells *in vivo* are only those cells of nonhematopoietic origin that are physically associated with maturing blood cells in the extravascular compartment of the bone marrow. The identity of marrow stromal cells *in vivo*, other than the conspicuous adipocytes and osteoblasts, has long remained as elusive as their morphology. For many years, the only advantageous way to image marrow stromal cells was with the use of scanning electron microscopy (SEM). The (pseudo) three-dimensional character of SEM images conforms well to the complex morphology of stromal cells. Using SEM, Weiss identified the adventitial reticular cells lying over the outer aspect of sinusoids. He also indicated that adventitial reticular cells could accumulate lipid and turn into marrow adipocytes (Weiss, 1976; Weiss and Sakai, 1984). In these pioneering studies, the "reticular" morphology was the only key to identification of the main cell type in the marrow stroma. However, a

"reticular" morphology can be shared by different cell types including macrophages. Westen and Bainton first demonstrated that the "reticulum" cells providing the physical substrate for medullary myelopoiesis were clearly distinguishable from macrophages and were characterized by membrane-bound alkaline phosphatase activity (Westen and Bainton, 1979). Subsequent studies demonstrated that identical cells are found in the human bone marrow (Figures 1*a*–1*e*), and in a variety of avian and mammalian species. In thin sections used for ALP cytochemistry, Westen-Bainton's reticulum cells appear as slender filaments (corresponding to cell processes) interspersed among hematopoietic cells. The use of organic reflective dyes for ALP cytochemistry and of three-dimensional confocal imaging of thick samples of human marrow conclusively established that Westen and Bainton's ALP-positive "reticulum" cells and Weiss' adventitial re-

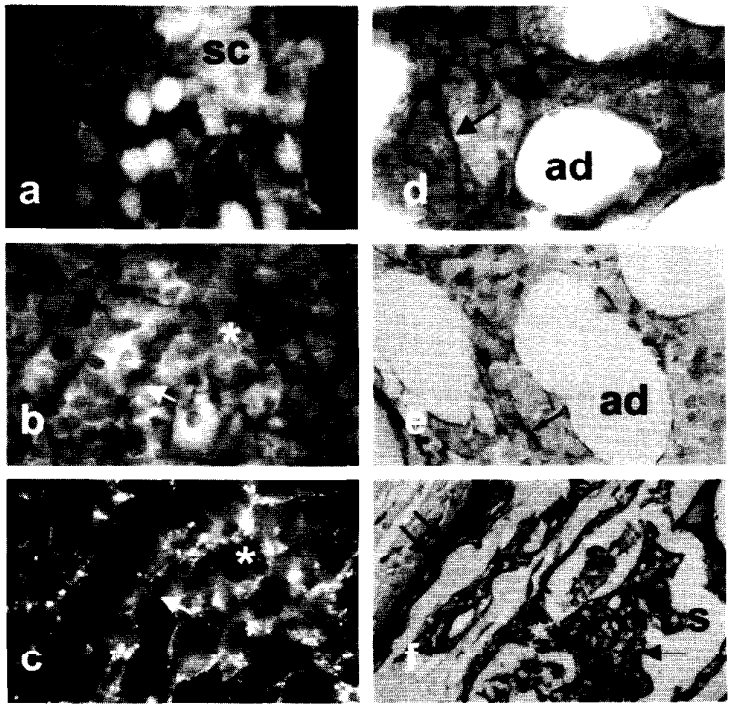

FIGURE 1. Marrow stromal cells *in vivo*. (*a*) Tandem scanning, reflected light, confocal imaging of human normal bone marrow, demonstrating a stromal cell (sc) extending over hematopoietic cells. Note the scanty amounts of thin, reticulin fibers. (*b*, *c*) Human leukemic marrow, thick sections stained for ALP activity as seen in transmitted light (*b*) and confocal reflection (*c*). Stromal cells appear as thin filaments (arrow) in transmitted light. Reflection provides a selective image of ALP-positive structures, with a wash-out effect excluding all hematopoietic cells. This demonstrates the system of spaces defined by stromal cells and accomodating hematopoiesis. (*d*, *e*) Thick (20 μm) and thin (2 μm) sections from low-temperature processed specimens of human marrow reacted for ALP, demonstrating stromal cells as filament-like structures interspersed among hematopoietic cells (ad, adipocyte). (*f*) Seventeen-day rat fetus, rib, ALP staining. Note the strong activity in the osteogenic cells of the periosteum (double arrow) and in the primitive, prehematopoietic, reticular marrow stroma (arrow) separating dilated sinusoids (s).

ticular cells are, in fact, the same (Bianco and Boyde, 1993).

In the postnatal human marrow, Westen-Bainton cells are clearly perivascular cells associated with the walls of small medullary arteries and sinusoids. They may rest directly on the abluminal side of the sinusoidal endothelium or project cell processes away from their surfaces and into the hematopoietic space proper.

The expression of the time-honored marker of osteogenic commitment *in vivo*, ALP, in marrow stromal cells did not escape Westen and Bainton's attention. Many have reasonably argued that the mere expression of ALP in reticular cells does not represent direct proof of kinship of these cells to the osteogenic lineage, as many stromal cells in culture, and most likely *in vivo* as well, may or may not be ALP positive. However, ALP expression is dynamically modulated even in bone cells proper—osteoblasts turn ALP activity off as they become osteocytes. In general, it should be remembered that there is no single phenotypic trait of the osteogenic lineage that is consistently retained throughout different developmental ages, maturational stages, and specific functions of a bone cell (reviewed in Gehron Robey et al., 1992). Stromal cells, likewise, can dynamically modulate well the expression of phenotypic markers (ALP, STRO-1, or any other marker) at the single cell level, as a mere result of functional adaptation.

Normally, the adventitia of sinusoids (where ALP-positive reticular cells are located) does not ossify, or the marrow space would turn into bone. This is precisely what happens, however, in egg-laying birds, in which estrogen-driven medullary bone occurs precisely at the sinusoid adventitia (reviewed in Turner, 1999). Notably, hematopoiesis is in part intravascular in birds, in which the extravascular marrow space transiently ossifies, and entirely extravascular in mammals. However, a pattern of perisinusoidal medullary ossification directly reminiscent of avian medullary ossification can be induced in rodents with colchicine (Arai et al., 1995), in conjunction with depletion of hematopoiesis. Additional circumstantial evidence links adventitial reticular cells and osteogenesis in diseases of the postnatal human bone marrow (reviewed in Bianco and Riminucci, 1998).

V. ONTOGENY AND LINEAGE OF MARROW STROMAL CELLS

Following the manner in which the marrow stroma develops in the context of bone development provides the appropriate angle for establishing lineage relationships within the so-called "stromal system." Any postulated lineage relationship should primarily accommodate the temporal sequence of events observed in development.

Bone marrow is a recent evolutionary acquisition, which follows the appearance of the bony skeleton. Bone marrow, and therefore its stroma, is established in developing bones only after a distinct bony collar has been formed and endochondral ossification proper has begun. This implies that both in phylogeny and ontogeny there is bone before there is marrow, and that fully competent osteogenic cells (osteoblasts) appear in development prior to (not after and not from) marrow stromal cells. The formation of a primitive marrow stroma in turn precedes the establishment of medullary hematopoiesis, whose existence directly depends on a stroma (Figure 1*f* and Figure 2). The primitive marrow stroma is established in the forming marrow cavity upon vascular invasion of the primitive bone anlage. The prehematopoietic marrow stroma is formed by "reticular" cells noted for their branched morphology, strong ALP activity, and active DNA synthesis (Bianco et al., 1993). These cells are physically continuous with, and phenotypically resemble, the primitive osteogenic, ALP-positive tissue that establishes the first bone (the bony collar). They surround the primitive sinusoid-like vessels that invade calcified cartilage to form the marrow cavity. The growth of vascular sprouts and perivascular ALP-positive osteogenic cells is coordinated, both in embryonic development and in its postnatal legacy, the advancing front of osteogenesis at the metaphyseal aspect of growth plates (Bianco and Riminucci, 1998; Rodionova, 1987; Rodionova and Skripchenko, 1986).

The primitive stroma is thus established as a local adaptation of the primary osteogenic cell population that initially develops in the periosteum. Concurrent with the ingrowth of osteogenic cells into the forming marrow cavity, the terminal vascular branches of the developing marrow spe-

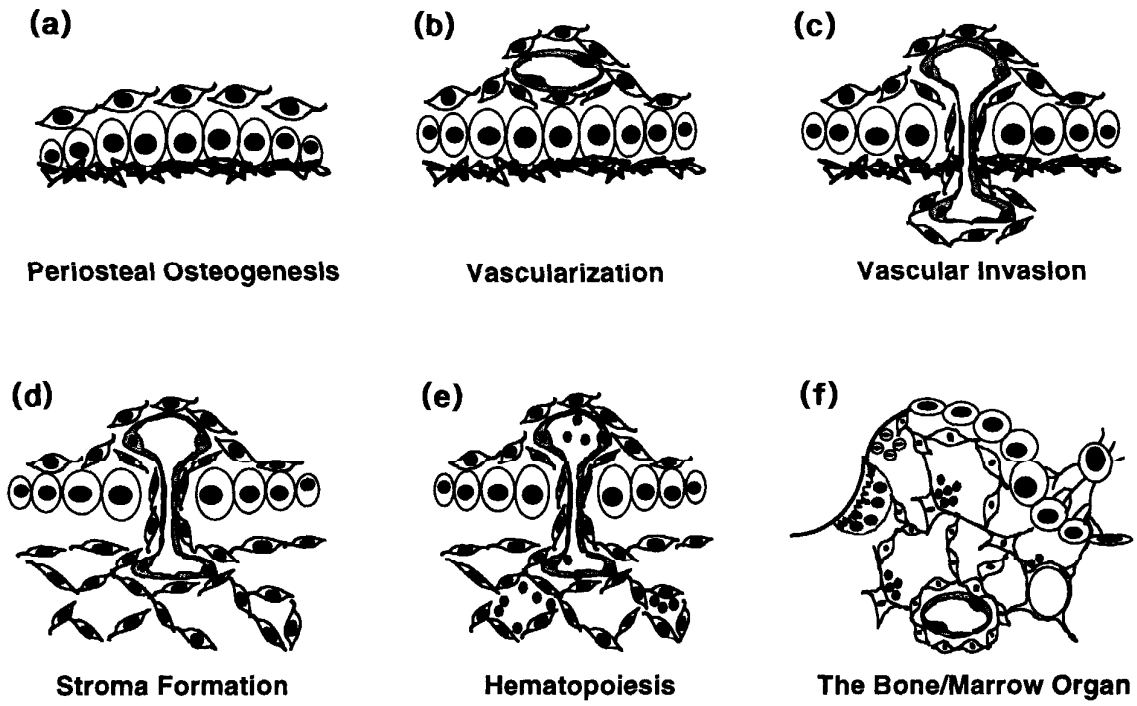

FIGURE 2. Diagram illustrating the development of the bone/bone marrow organ. Development of the bone marrow stroma is preceded by development of a bony collar by committed osteogenic cells in the periosteum that surrounds the developing rudiment (*A*). Committed osteoprogenitors associate with the surfaces of blood vessels that grow into this newly formed tissue (*B*). As the blood vessels invade the cartilaginous interior of the developing rudiment, the osteoprogenitors are carried along (*C*) and continue to proliferate to keep pace with the development of the vascular sprouts, thereby establishing the primitive stroma (*D*). Subsequently, hematopoietic stem cells leave the vasculature and associate with the stroma, thereby establishing extravascular hematopoiesis (*E*). After birth, when hematopoiesis is sufficient to support the organism, the excess stroma forms space-filling adipocytes. This complete bone/bone marrow organ can be seen as a continuous network of osteogenic and bone lining cells, myelosupportive stroma, and adipocytes (*F*).

cialize to give rise to the system of sinusoids. These are characterized by a large caliber and a slow blood flow, by the lack of a continuous physical basement membrane, and by the ability of their endothelial cells to allow for transcellular migration of cells (reviewed in Tavassoli and Yoffey, 1983). These characteristics are putatively permissive for the migration of blood-borne hematopoietic stem cells into the extracellular space of the developing marrow. Here, the interaction of hematopoietic stem cells with the osteogenic cells forming the primitive stroma leads to hematopoietic stem cell commitment, differentiation of blood cell precursors, and the establishment of hematopoiesis (Figure 1). As a result, the osteogenic potential of the stromal cells is in some way kept at bay, allowing for the formation of conspicuous marrow spaces intertwined within a trabecular bony architecture, instead of a continuous bony phase. Mechanisms underlying this putative negative regulation of the osteogenic potential of primitive stromal cells are unknown, but noggin, a negative regulator of BMP signaling effects (Francis-West et al., 1999), and the notch/notch ligand systems (Varnum-Finney et al., 1998) are natural candidates deserving specific attention. Capture of space for excessive, pathologically expanded hematopoietic tissue may result in severe changes in the architecture of bone. These can be observed, for example, in congenital hemolytic anemia, in which a compensatory expansion of marrow erythroid precursor

cells turns compact bone into trabecular bone, and trabecular bone into porotic bone (Ascenzi, 1976). Once hematopoiesis is established, stromal cells stop DNA synthesis and may enter a G_0 phase lasting for the entire lifespan of the organism (A. J. Friedenstein, personal communication).

VI. REGULATION OF OSTEOGENIC COMMITMENT

Recently, a transcription factor, *cbfa1/AML-3*, has been identified as a major regulator of osteoblastic differentiation (Banerjee et al., 1997; Ducy et al., 1997; Stein et al., 1998). Bone marrow does not develop if primary osteogenic differentiation is blocked in mouse embryo by ablation of factor *cbfa1/AML-3*, whereas differentiation of other mesenchymal tissues (cartilage and fibrous tissue) that can be derived from the "stromal system" is not impaired (Komori et al., 1997; Otto et al., 1997). *Cbfa1*-deficient mice do not form bone, their cartilage rudiments remain uninvaded, and no marrow stromal cells are formed. Spontaneously immortalized mouse stromal cell lines derived from postnatal mouse marrow constitutively express *cbfa1* transcripts, regardless of their ability to express osteogenic phenotypic traits in culture or, more significantly, to establish an ectopic "ossicle" upon *in vivo* transplantation in the subcutis of immunocompromised recipient animals (Satomura et al.). Interaction of *cbfa1* with a variety of additional factors may, in principle, result in modulation of the effects of *cbfa1* on osteogenic differentiation (Chen et al., 1998). The significance of these modulatory interactions may be especially relevant to models mimicking instances of osteogenesis other than the primary embryonic bone formation, which is blocked by ablation of *cbfa1*. Human marrow stromal cells isolated in culture from postnatal organisms also constitutively express multiple isoforms of CBFA1. Notably, the human homologue of the unique N-terminal sequence found in the mouse osf2 protein and responsible for binding to the OSE2 element in the mouse osteocalcin gene is not transcribed (Xiao et al., 1998). And, as in the mouse, as noted above, CBFA1 expression in human stromal cells does not correlate to osteogenic differentiation in culture or in *in vivo* transplantation assays.

The significance of CBFA1 expression in postnatal stromal cells with respect to their ability to progress to complete osteogenic differentiation thus remains to be determined. Indeed, at this time, the overall significance of CBFA1 in dictating the differentiation and maturation of *postnatal* osteoblasts involved in adult remodeling may be diminished from its role in *primary* embryonic osteogenesis. By analogy, the role of other transcription factors belonging to the runt homology family in mouse hematopoiesis, for example, is obviously linked to specific temporal and organ-specific stages of hematopoiesis. It has been known for decades that primary embryonic bone and postnatal secondary bone differ in structure. However, the differences in the molecular pathways by which primary and secondary osteogenesis are regulated have never been formally addressed, although evidence indicates, for example, distinct profiles of hormonal responsiveness, and matrix protein expression and deposition by embryonic and postnatal osteoblasts.

In view of the apparent high level of restriction of CBFA1 expression to osteogenic cells in development, the expression of CBFA1 in marrow stromal cells provides the strongest marker of a lineage relationship of postnatal stromal cells to the osteogenic lineage available to date. CBFA1 expression also indicates that stromal cells that we are currently able to isolate in culture express the "master gene" of osteogenic commitment. The stromal cells that we are able to identify as in situ tissue components, on the other hand, express the time-honored *in vivo* marker of osteogenic commitment, alkaline phosphatase. We take this data as evidence that postnatal stromal cells bear the imprint of osteogenic commitment, which took place earlier on in development, and was inherited in a population of cells remaining in a prolonged G_0 phase.

VII. STROMAL CELLS *IN VITRO*

The stromal cell *in vitro* is defined as the rapidly adherent population that arises from the clonogenic growth of individual cells, the CFU-F,

and has none of the cardinal features of endothelial cells and macrophages (Friedenstein, 1990; Kuznetsov and Gehron Robey, 1996; Kuznetsov et al., 1997a). Upon closer inspection of individual colonies that are formed by these CFU-F, several traits that are characteristic of the stromal cell system *in vivo* appear to be somewhat segregated into individual colonies, with some colonies dedicated to osteogenesis (production of alkaline phosphatase, other bone-related proteins, and accumulation of calcium) and adipogenesis (the expression of adipogenic markers and fat accumulation), and others with no particular phenotype (but perhaps indicative of myelosupportive or fibrogenic phenotypes) (Figure 3). When plated at high cell densities, the adherent BMSC population displays osteogenic, adipogenic, and myelosupportive character (Dorheim et al., 1993; Hangoc et al., 1993). After passaging of such a mixed population of colonies, the cells revert to a less obvious phenotype (Bruder et al., 1997; Kuznetsov and Gehron Robey, unpublished results). However, they maintain their ability to differentiate into the relevant phenotypes by altering the tissue culture conditions in which they are maintained.

Induction of the osteogenic phenotype *in vitro* requires time, and can be enhanced by the addition of modulators to the culture medium. As demonstrated previously in cultures of more mature osteoblastic cells, cells progress through different stages of maturation that are characterized by a proliferative phase, followed by a somewhat phase-specific production of extracellular matrix proteins. Cultures that are maintained in the presence of dexamethasone and ascorbic acid (Herbertson and Aubin, 1997; Kuznetsov et al., 1997a,b; Rickard et al., 1994), or after exogenous treatment with BMPs (Thies et al., 1992), will initiate osteogenic events as indicated by the synthesis of bone matrix proteins such as bone sialoprotein and osteocalcin that characterize the early stages of matrix mineralization. Addition of 1,25-dihydroxy vitamin D_3 (Beresford et al., 1992; Leboy et al., 1991; Rickard et al., 1994), or other factors such as estrogen (Benayahu, 1997; Shamay

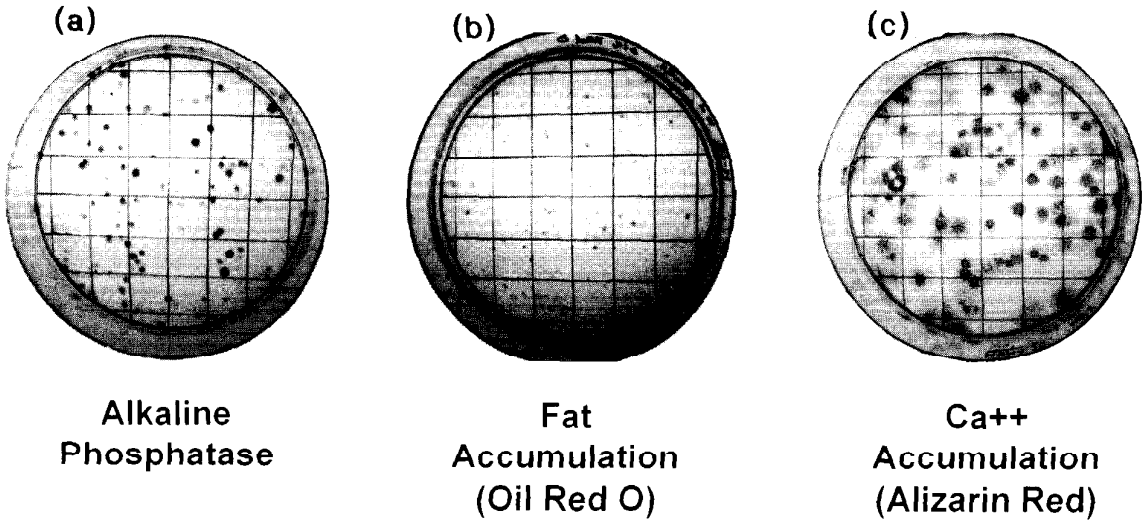

FIGURE 3. Phenotypic heterogeneity in the progeny of CFU-F *in vitro*. When bone marrow cell suspensions are plated at low density, individual CFU-F adhere and proliferate to generate a colony. These colonies are found to have different phenotypic characteristics representative of the stromal cell system *in vivo*. Some colonies are alkaline phosphate positive (*A*), which can be indicative of the osteogenic, myleosupportive, or preadipogenic phenotype, whereas others are found to contain lipid (as determined by Oil Red O) staining, indicative of more mature adipocytes (*B*). With continued culture, many colonies form nodules and accumulate calcium (alizarin red staining), characteristic of more mature osteoblastic cells (*C*).

et al., 1996; Zhang et al., 1995), TGF beta super family members (Benayahu et al., 1993; Gazit et al., 1993; Gordon et al., 1997), IL-1, TNF-alpha, and members of the IL-6 cytokine family (Romas et al., 1996) also positively effect osteoblastogenesis when added to the culture medium at different times. The end result of these types of regulators *in vitro* is the establishment of a so-called "bone nodule," which accumulates calcium as detected by von Kossa staining. However, these types of nodules may not always be indicative of osteogenesis and, in fact, true bone formation, as characterized by appropriate architecture, must rely on *in vivo* transplantation (Krebsbach et al., 1997).

VIII. ADIPOCYTES, THE STROMA, AND BONE

Primed by earlier observations (Meunier et al., 1971), a bulk of current literature deals with potential "common progenitors of osteoblasts and adipocytes," which would explain some aspects of age-related bone loss. Most of these studies directly or indirectly imply that osteogenic and adipogenic differentiation normally occurs continuously and at a high rate in bone, regardless of any specific physiological circumstance. However, this is not the case.

Adipocytes are not found in the fetal marrow. They develop postnatally, as bone growth progressively makes further enlargement of the marrow space corresponding to the needs for hematopoiesis. At the end of skeletal growth, more space becomes unnecessary because of the progressive, age-dependent shrinkage of the hematopoietic cell mass that is accompanied by formation of adipocytes. Conversely, any increase in hematopoietic cell numbers that occurs in the adult life, as dictated by homeostatic mechanisms or disease (e.g., leukemia), leads to the loss of adipocytes to accommodate the expanded hematopoietic tissue (Bianco and Riminucci, 1998). In children, where sustained expansion of the hematopoietic cell mass cannot occur at the expense of adipocytes, hematopoiesis expands at the expense of bone tissue. Obvious changes in the number of adipocytes, as dictated by changes in hematopoiesis in adult life, are rapidly detectable. Acute loss of hematopoietic cells in the marrow is accompanied by rapid and sustained adipogenesis (Figure 4). This occurs very efficiently in the bone marrow even against heroic antimitotic regimens, such as those used in leukemia or in the pre-BMT conditioning. This suggests that cell division is not required for adipocyte development, consistent with a direct conversion of adipocytes from a local resident cell capable of accumulating lipid. The direct formation of marrow adipocytes from marrow reticular cells has been documented in different species and is best illustrated in humans by changes that follow rapid depletion of the hematopoietic cell numbers. Here, direct conversion of ALP-positive (WB) cells can be observed at the time of maximal depletion of in-cycle cells, suggesting that no cell division is involved in the genesis of marrow adipocytes *in vivo*. As an ALP-positive cell accumulates lipid, ALP activity is progressively decreased, leading to an ALP-negative adipocyte (Bianco et al., 1988). A similar pattern of coordinated adipo-genesis and downregulation of ALP activity is mimicked in cultures of murine marrow fibroblasts (Kodama et al., 1983).

The peroxisome proliferator-activated receptor-gamma (PPARgamma) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors. It is predominantly expressed in adipose tissue, but also in other tissues such as the adrenal gland and spleen. PPARgamma has been shown to regulate adipocyte differentiation in extraskeletal connective tissues, and is regarded as a potentially major player in adipogenesis in marrow stromal cells as well. PPAR gamma1 and PPARgamma2 regulate the expression of adipocyte-specific genes, such as the fatty acid binding protein, aP2, and adipsin. PPAR gamma ligands include structurally varied drugs, such as the antidiabetic thiazolidinediones, and natural ligands such as fatty acids and PGJ2 (Brun et al., 1997; Smas and Sul, 1997; Spiegelman, 1998). PPARgamma is expressed in marrow stromal cells and mediates their thiazolidinedione-induced adipose conversion. However, PPAR gamma expression is not strictly required for obtaining adipogenesis *in vitro*. Cells that do not express PPARgamma may still accumulate fat

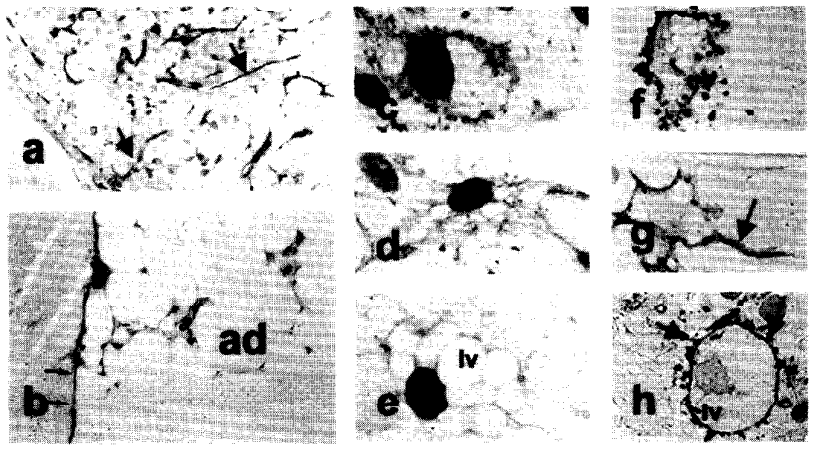

FIGURE 4. Adipogenesis in the human postnatal marrow. (*a*) Rich network of Westen-Bainton cells in a hypercellular (leukemic) marrow before myeloablation. (*b*) Marrow from the same patient following myeloablation. Depletion of hematopoietic cells is accompanied by depletion of WB cells; the marrow is occupied by adipocytes. (*c–h*) Human bone marrow. Adipose conversion of ALP-positive stromal cells at the time of maximal cell depletion induced by myeloablation (14 days of chemotherapy). Cells accumulating lipid (*c–e*) express ALP activity on their plasma membrane (lv, lipid vacuoles).

upon treatment with appropriate "cocktails." In some systems, this effect may be mediated by other members of the PPAR family, such as PPARalpha, PPARdelta, or NUC-1 (reviewed in Gimble et al., 1996). Members of the PPAR family may be activated by different ligands, depending on the cell system. Thiazolidinediones bind to, and induce the transcription of PPARgamma, while not affecting, or even depressing, mRNA levels for other PPAR family members (Lambe and Tugwood, 1996). Long chain fatty acids may, in contrast, act as the main activators of PPAR factors other than PPARgamma in cells that do not express it. The high content of long chain fatty acids in rabbit serum may be related to its adipogenic effect and perhaps to the adipogenic effect of sera from other species (e.g., horse serum, individual batches of bovine serum) (Diascro et al., 1998).

PPARgamma is involved in functions other than adipogenesis in unrelated cell types. It is expressed in hematopoietic cells and macrophages, and is highly upregulated in activated macrophages. Binding of PGJ2 metabolites in activated macrophages leads to active repression of genes involved in the inflammatory response via interference with AP-1, STAT, and NF-kappaB transcription factors (Ricote et al., 1998). Hypothetically, ligand-activated PPARgamma may thus contribute to both positive and negative regulation of gene expression in cells of the stromal system. Concurrent repression and activation of sets of genes involved in separate differentiation pathways may be a mechanism underlying the plasticity of stromal cells.

Commitment of stromal cell cultures to the adipogenic phenotype has been noted following the addition of a particular "cocktail" (hydrocortisone, isobuteryl methyl xanthine, and indomethacin) or following treatment with the antidiabetic compounds, the thiazolidinediones (Gimble et al., 1996; Gimble et al., 1994). In addition, changing the type of serum that is used in the culture medium also gives rise to adipocytic cultures, apparently due to differences in fatty acid content (Beresford et al., 1992; Deryugina and Muller-Sieburg, 1993; Diascro et al., 1998; Lanotte et al., 1982). In general, factors that activate PPARgamma or induce CAAT/enhancer binding protein (C/EBP) (a transcription factor essential for adipogenesis), such as

fatty acids and prostaglandins, induce adipogenesis (reviewed in Gimble et al., 1996). *In vitro*, dexamethasone has been noted to increase the expression of osteoblastic markers, contrary to its well-known effect of inducing bone loss *in vivo*. However, pharmacological doses of dexamethasone may also induce adipocyte formation (Cui et al., 1997). The role of 1,25-dihydroxyvitamin D_3 in adipogenesis is also unclear in that it has been reported to induce adipocyte formation when added alone or simultaneously with dexamethasone (Grigoriadis et al., 1988), or to block adipogenesis induced by the cocktail of hydrocortisone, IBMX, and indomethacin (Kelly and Gimble, 1998). Members of the TGF-beta superfamily also have pleiomorphic effects, with low concentrations being inductive and higher concentrations being inhibitory (Asahina et al., 1996).

IX. ADAPTATION AND CHANGE VS. CONSUMPTION AND REPLACEMENT

The hematopoiesis-supporting stroma originates from cells that were once committed to osteogenesis. Adipocytes originate postnatally from cells that were once hematopoiesis-supporting stroma (Figure 5). These members of the stromal system, when seen in a lineage, are metachronous, not synchronous. A precise temporal sequence scans their appearance in bone as an organ, which obeys precise developmental or growth-related adaptive changes. Some of them do not express one sole phenotype. In culture, adipocytes can be reverted to a fibroblast-like morphology and further induced to form bone *in vivo* (Bennett et al., 1991). This is an example of the "plasticity" of the stromal system, that is, the ability of differentiated stromal cells to shift phenotype.

Consumption and replacement with maintenance of a steady-state organ size is the inherent dynamic of blood or of the epidermis, the two best-characterized systems dependent on a stem cell. A stem cell of the hematopoietic/epidermis type may have evolved as an advantage for organisms outliving cells performing critical vital functions. Plasticity is a recognized inherent characteristic of the stromal system, which may have evolved to ensure the adaptability of mesodermal

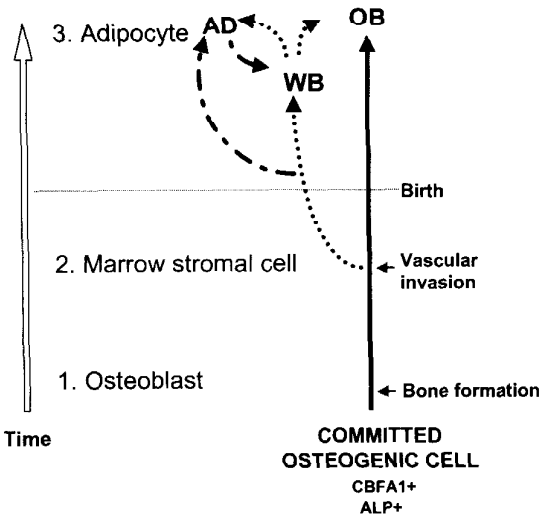

FIGURE 5. Emergence and mutual dependence of the main postnatal stromal phenotypes (reticular cells, osteoblasts, and adipocytes) as a function of time and adaptation. During development, committed osteogenic cells, which are alkaline phosphatase positive and also express the "master gene" of osteoblastic differentiation (CBFA1), appear prior to other stromal phenotypes. These cells form full mature osteoblasts, which deposit bone on the surface of the developing rudiment. Marrow stromal cells, which are also alkaline phosphatase positive, later evolve downstream of osteogenic commitment. After birth, at which time adequate levels of hematopoiesis have been generated to support the organism, some stromal cells lose alkaline phosphatase positivity and accumulate fat to form adipocytes. In the postnatal organism, stromal cells express CBFA1 constitutively and can dynamically modulate the osteoblastic, myelosupportive, and adipocytic phenotypes.

tissues themselves. This is required for the coordinated growth of complex organs comprised of different tissues, such as individual bones. Adaptation and growth is the dominating, evolutionarily conserved dynamic of mesodermal tissues. The molecular mechanisms underlying the stromal/mesodermal plasticity may thus be quite distinct from mechanisms of commitment and differentiation of other systems, as they have to accommodate reversibility and phenotypic shifts. Obviously, control of differentiation based on association with extracellular matrices is an important aspect of connective tissue biology. This

implies the environmental selection of arrays of transcription factors dictating different differentiation pathways within a single cell, but does not necessarily imply an undifferentiated state of the candidate cell. In the age of Dolly, one should try to see cell differentiation and lineage in a more modern, flexible way, of which the stromal system may provide a paradigm. As the dissection of molecular mechanisms underlying the biology of stromal progenitors is undertaken, one approaches the biology of an individual cell's flexibility in complex long-lived organisms.

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